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MODULATION OF THE METABOLISM AND CYTOTOXICITY OF THIOPURINES IN L1210 CELLS BY METHOTREXATE PRETREATMENT

PAUL M. SNYDER

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MODULATION OF THE METABOLISM AND CYTOTOXICITY OF THIOPURINES IN L1210 CELLS BY METHOTREXATE PRETREATMENT

Paul M. Snyder

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Introduction

Methotrexate (MTX) and 6-thioguanine (6-TG) are antimetabolites used both singly and in combination for the treatment of various forms of leukemia and other neoplasms (1,2). The mechanism of action of MTX has been extensively investigated. By inhibition of the cellular enzyme, dihydrofolate reductase, MTX causes a depletion of reduced folates which are necessary for a number of biosynthetic reactions including de novo purine and thymidylic acid synthesis (1); Figure 1 illustrates this effect. In the cell this depletion of the thymidine and purine deoxynucleotides leads to suppression of DNA synthesis and ultimately to cell death. If cells are provided with an exogenous source of purines such as inosine or hypoxanthine and with exogenous thymidine, the cytotoxicity of MTX is circumvented. Another indirect metabolic consequence of MTX treatment is an increase in the intracellular concentration of phosphoribosylpyrophosphate (PRPP) due to the suppression of purine biosynthesis. By itself, the expansion of PRPP pools is of little consequence, but it can increase the rate of metabolism of other molecules which utilize PRPP as a cofactor. In the case of 5-fluorouracil (FUra), a cytotoxic agent which is activated by conversion of FUra to FUra-ribosephosphate using PRPP and orotate phosphoribosyltransferase, it has been shown that pretreatment of cells with MTX leads to increased activation of FUra and enhanced cytotoxicity (3,4). Thus MTX treatment, in addition to its inherent toxicity, can metabolically increase the effectiveness of certain other agents leading to synergistic cytotoxicity.

The mechanism of 6-TG toxicity is much less well established. It is converted in the cell to 6-TG ribosephosphate (6-TGRP) by the enzyme hypoxanthine-guanine phosphoribosyltransferase using PRPP as the cofactor; this

conversion is apparently necessary for the cytotoxic action of 6-TG. While 6-TGRP has been shown to inhibit a number of enzymes involved in purine biosynthesis, it has been reported that the primary mechanism of cytotoxicity of 6-TG is via its incorporation into DNA (5). Incorporation of 6-TG into RNA has been reported, but no connection between this incorporation and cell toxicity has been shown. Since 6-TG, like FUra, is activated by reacting with PRPP (1,2), it seemed possible that 6-TG and MTX might have a synergistic interaction analogous to that already demonstrated between MTX and FUra.

The present study characterizes cytotoxic and metabolic interactions between MTX and 6-TG in L1210 mouse leukemia cells in culture. It is shown that there is indeed a modulation of 6-TG activity by MTX.

Materials and Methods

Chemicals. (^{14}C)6-TG was purchased from Moravek Biochemicals (City of Industry, CA). MTX was purchased from Lederle Laboratories (Wayne, NJ). All other biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO). Tissue culture supplies (unless otherwise designated) were obtained from Gibco Laboratories (Grand Island, NY).

Cells. L1210 murine leukemia cells were maintained in suspension culture with Fischer's medium supplemented with 10% heat-inactivated horse serum at 37°C in a 5% CO_2 atmosphere. All experiments were performed using logarithmically growing cells (1 to 5 x 10^5 cells/ml) which had been initially seeded at 1 to 3 x 10^4 cells/ml. Cell density was determined with the use of a model ZBI Coulter Counter (Coulter Electronics, Hialeah, FL).

Cloning. Cell viability and drug toxicity were determined using the soft-agar cloning assay as described by Cadman et al. (3). Following the indicated single drug exposure to logarithmically growing cell cultures, the second drug was added for the specified time. The drug-containing medium was then removed after centrifugation at 1000 x g for 5 min at 37°C. The cells were resuspended in drug-free medium and recentrifuged as before. This washing procedure was repeated twice to remove any extracellular drug before cloning. Fifty cells contained in 2 ml of liquified agar 37°C and 3 ml of drug-free Fischer's medium plus 15% horse serum were pipetted into 10 ml culture tubes. The tubes were capped, placed in an upright position and incubated at 37°C in a 5% CO_2 atmosphere. The amount of agar in the culture medium was 88 mg/100 ml; the consistency of this mixture allows cell growth without cell settling. Cells that remain viable after drug exposure, as defined by the capability to divide and produce progeny, will form individual cell colonies after 10 days of incubation. All clones were counted with an inverted microscope. Single cells, which can be visualized in suspension cultures were not observed in the cloning medium after the 10 day incubation, indicating that all viable cells had developed into clones. The percent viability is the ratio of clones formed from drug-treated cultures to clones formed from untreated cultures, multiplied by 100. The cloning efficiency of L1210 cells in this system was 85 to 90%. All data shown are the mean of triplicate experiments performed on 3 separate occasions. The variation between experiments was less than 10%. Drug concentrations were measured by spectrophotometric analysis and drug solutions were prepared the same day of use.

6-TG Metabolism. Cells were exposed to (^{14}C)6-TG for time periods from 1 to 180 min. At the specified time, iced saline containing 50 μM dipyridamole was added; the cell pellet was immediately isolated by centrifugation and then disrupted by the addition of ice-cold 0.2 N $HClO_4$. To a tube which contained the

iced cells (^{14}C)6-TG was added as a control to determine the amount of non-specific radioactivity that would persist during the following DNA and RNA isolation procedures. The acid-soluble fraction was isolated for HPLC analysis of intracellular 6-TG nucleotides. The RNA and DNA were isolated from the acid-precipitate as follows: The acid-precipitate was washed twice with iced 0.2 N $HClO_4$, followed by the addition of 3 ml of 0.3 N NaOH to the washed pellet. The precipitate was then digested for 1 hour at 37°C. After cooling on ice, DNA and protein were precipitated by the addition of 300 µl 5.2 N $HClO_4$. Five hundred microliters of the isolated supernatant (containing the RNA fraction) was measured for radioactivity. This process was repeated twice to insure complete removal of all RNA from the remaining precipitate. Following removal of the RNA-containing supernatant, the precipitate was washed twice with iced 0.2 N $HClO_4$, resuspended in 3 ml of 0.5 N $HClO_4$, and incubated at 70°C for 30 min to solubilize the DNA fraction. After cooling on ice, the sample was centrifuged and 500 µM of the supernatant (containing the DNA fraction) was measured for radioactivity. The precipitate was then washed twice with 0.5 N $HClO_4$ and digested for 1 hour at 70°C in 3 ml of 0.5 N KOH. Five hundred microliters of the supernatant (containing the protein fraction) was neutralized with 1 N HCl and measured for radioactivity. Total RNA was quantitated by the orcinol reaction, and total DNA was quantitated by a standard diphenylamine procedure (6). HPLC analysis of acid-soluble pools was carried out on a SAX anion-exchange column using a 60 min linear gradient from 0.01 M potassium phosphate (pH 5.7) to 0.75 M potassium phosphate (pH 4.7).

Cesium Sulfate Gradients. Incorporation of (^{14}C)6-TG into nucleic acid was studied using the method of Kufe et al. (7) for quantitative separation of RNA and DNA by Cs_2SO_4 equilibrium gradient centrifugation. After the indicated drug exposures to exponentially growing cultures, iced saline containing 50 µM

dipyridamole was added and the cell pellet was isolated by centrifugation. Cells were resuspended at a density of 10^7 cells/ml in PBS, and 1 ml of the suspension was added to 2 ml of a solution containing 1.25 mg/ml pronase B (self-digested for 15 min at 37°C), 0.01 M Tris, pH 7.4, 1 mM EDTA, and 0.5% SDS. The mixture was allowed to digest for 3 hours at 37°C and the total nucleic acid was isolated by 3 cycles of phenolchloroform extraction. Nucleic acid was precipitated at -20°C overnight in 2 volumes of absolute ethanol in 0.4 m NaCl. The pellet isolated by centrifugation was resuspended in 0.5 ml of 10 nM Tris, pH 8.0, 10 mM EDTA; and equal volume of formamide was added and the mixture was incubated 15 min at 80°C. To the mixture was then added 4.14 ml saturated Cs_2SO_4, 3.36 ml 5 mM EDTA; the resulting solution was then centrifuged in a Beckman 50 Tri rotor at 40,000 rpm for at least 60 hours. The gradients were collected in about 50 fractions of equal volume from the bottom of the tube; the trichloroacetic acid-precipitable material in each fraction was collected using a MASH multi-sample processor (Microbiological Associates, Walkersville, MD) and the radioactivity measured in a Packard Tricarb liquid scintillation spectrometer.

Results

Cytotoxicity. The cytotoxicity of 6-TG alone and in combinations with MTX was studied and the results are summarized in Figure 2. This graph shows the clonal growth of L1210 mouse leukemia cells following either a 3 hr exposure to 6-TG, a 3 hr exposure simultaneously with 1 μM MTX and 6-TG, or a 3 hr exposure to 6-TG following a 3 hr pretreatment with 1 μM MTX. Treatment with 1 μM MTX alone for 3 hr resulted in a 52% inhibition of clonal growth compared to control, as depicted in Figure 2 by the horizontal dotted line. Exposure of L1210 cells to 6-TG

for 3 hr led to a dose-dependent inhibition of clonal growth with an LD_{90} of 0.8 μM. Pretreatment of cells for 3 hr with 1 μM MTX followed by a 3 hr treatment with 6-TG resulted in a synergistic inhibition of clonal growth at all concentrations tested; the LD_{90} for 6-TG was about 10 nM with 1 μM MTX pretreatment. In contrast, when L1210 cells were exposed simultaneously to 6-TG and 1 μM MTX for 3 hr, there was no enhancement of 6-TG cytotoxicity. At low concentrations of 6-TG (1-10 nM), the inhibition of clonal growth was at best additive; at higher concentrations of 6-TG (0.1-1 μM), the 1 μM MTX was actually antagonistic to 6-TG cytotoxicity. Thus, the modulation of 6-TG cytotoxicity can be either positive or negative. When 6-TG exposure follows MTX pretreatment, there appears to be a synergistic enhancement of 6-TG cytotoxicity, but with concurrent MTX and 6-TG exposure there is an apparent negative modulation or antagonism of 6-TG cytotoxicity.

Cellular Uptake of 6-TG. To determine the effect of MTX pretreatment on total cellular uptake of 6-TG into acid-soluble intracellular pools, cells were exposed to 1 μM (^{14}C)6-TG either with or without pretreatment of the cells with 1 μM MTX; samples were removed from each culture at times ranging from 1 to 150 min, and the PCA-soluble radioactivity was determined as described in Methods. The results, normalized to the amount of ribose (as RNA) in the acid-precipitable fraction of each sample, are shown in Figure 3. The uptake of (^{14}C)6-TG into L1210 cells is rapid, with apparent equilibration of the acid-soluble pool in less than five minutes; since the only earlier sampling was at one minute, no statement can be made comparing the relative rates of 6-TG uptake in MTX-pretreated versus control cells. There was a small difference in the steady-state concentration of 6-TG in acid-soluble intracellular pools. It is important to keep in mind that the acid-soluble fraction contains not only free 6-TG but all the small metabolites of 6-TG, including 6-TG nucleotides. MTX-pretreated cells had a

steady-state level of about 2.9 pmol 6-TG per pmol cellular RNA ribose, compared to about 2.5 pmol 6-TG per pmol cellular RNA ribose in control cells.

Metabolism of 6-TG. As demonstrated above, MTX pretreatment resulted in increased uptake of 6-TG into the acid-soluble fraction. The composition of the acid-soluble pools was further characterized by HPLC analysis as follows. Cells were exposed to 0.5 μM (^{14}C)6-TG for 60 min with and without a 3 hr pretreatment with 1 μM MTX. The acid-soluble fraction of the cells was isolated and anlayzed using HPLC as described in Methods. This allowed determination of the intracellular levels of free 6-TG as well as the mono-, di-, and triphosphate metabolites. These results are shown in Table 1. While the intracellular concentration of free 6-TG was almost identical in MTX-pretreated and control cultures, there was a substantial difference in the levels of nucleotide metabolites. TGMP, TGDP, and TGTP levels were all higher in MTX-pretreated cells than in control cells. The total 6-TG nucleotide concentration, in pmol per 10^6 cells, was 2.40 in the MTX-pretreated culture, compared to 1.55 in the control cells. MTX pretreatment appears to result in an enhancement of the intracellular conversion of 6-TG into its nucleotide derivatives.

Incorporation of 6-TG into RNA and DNA. The effect of MTX on 6-TG incorporation into cellular nucleic acids was investigated. Two standard methods were used to quantitate the relative incorporation of 6-TG into RNA and DNA. The first method distinguished RNA and DNA incorporation on the basis of differential hydrolysis by mild alkaline and hot acid conditions. The second method, used to confirm the results obtained by the chemical procedure, separated DNA from RNA using cesium sulfate density gradients (see Methods). Figure 4 shows the time course of incorporation of (^{14}C)6-TG into RNA, with and without a 3 hr pretreatment with 1 μM MTX, using the chemical method. As expected from the higher levels of 6-TG nucleotides measured in MTX-pretreated cells, there was

a marked increase in the rate of (^{14}C)6-TG incorporation into RNA when cells were pretreated with 1 μM MTX. The effect of MTX pretreatment on 6-TG incorporation into DNA was quite different. There is a dramatic suppression of 6-TG incorporation into DNA after a 3 hr pretreatment with 1 μM MTX, as shown in Figure 5.

The results obtained in experiments of similar design which used cesium sulfate equilibrium density gradients as the technique for separating RNA from DNA are shown in Figures 6 and 7. The results are qualitatively similar to those obtained by the differential hydrolysis procedure: MTX pretreatment at 1 μM for 1 hr leads to enhanced incorporation of (^{14}C)6-TG into RNA while suppressing incorporation of (^{14}C)6-TG into DNA. While there are quantitative differences in the results which are probably attributable to the inherent differences in the technique, there is good qualitative agreement. This lends independent support to the conclusions drawn using the other technique.

Discussion

These experiments have demonstrated that MTX can modulate the cytotoxic activity of 6-TG in mouse L1210 cells, and that this modulation depends on the schedule of drug administration. By characterizing some of the biochemical interactions of MTX and 6-TG in L1210 cells, these studies provide evidence for the metabolic basis of the drug interaction and suggest a mechanism to explain the synergistic cytotoxicity found under some conditions.

Pretreatment of L1210 cells with MTX synergistically enhanced the cytotoxic activity of 6-TG as measured by the soft-agar cloning assay. The same MTX pretreatment was found to result in somewhat increased uptake of 6-TG into cells,

increased conversion of free 6-TG to 6-TG nucleotides, and markedly increased incorporation of 6-TG into RNA. Simultaneous treatment with MTX and 6-TG resulted in an antagnostic interaction between the drugs. An analogy can be drawn between these results and those already reported describing the interaction between MTX and 5-FU (3,4,8). The cytotoxicity of 5-FU is synergistically enhanced by MTX pretreatment of murine L1210, human colorectal tumor line HCT-8, and human breast carcinoma line 47-DN (3,8); in L1210 cells, simultaneous MTX and FUra treatment antagonizes the FUra cytotoxicity. It was shown (4) that the enhanced FUra toxicity in MTX-pretreated cells was due to increased intracellular levels of PRPP, the co-substrate of orotate-phosphoribosyltransferase which activates FUra in the cells; the increased availability of PRPP leads to enhanced activation of FUra to FUra-nucleotides and thus greater cytotoxicity. A 3 hr treatment wtih 1 μM MTX, the regimen used in these experiments, was found to result in a 4- to 5-fold expansion of PRPP pool size; this was associated with a marked increase in the level of FUra-nucleotides and in the rate of FUra incorporation into RNA. These experiments with 6-TG are less complete in a number of ways, but nevertheless they demonstrate important points. The effect of a varying MTX dose on cytotoxicity or on the various biochemical parameters was not examined; we cannot say that the dose of 1 μM was optimal or that the pretreatment interval of 3 hr was ideal for modulation of 6-TG activity. Therefore, a dose-response relationship has not been established between MTX pretreatment (thus by inference cellular PRPP levels) and cellular 6-TG nucleotide levels or 6-TG incorporation into RNA. Noteworthy, however, is the recent report (9) of other work in this laboratory that a 3 hr exposure of L1210 cells to 1 μM MTX increased intracellular PRPP levels from 4.1 to 125 ngm/10^6 cells. This demonstrates a correlation between elevated PRPP levels and the increased activation of 6-TG, reported here, following pretreatment for 3 hrs with 1 μM

MTX. In the present report, 6-TG concentration was held constant in all experiments except the cytotoxicity assays, so the effect of varying 6-TG concentration or treatment interval is not known. These provisos notwithstanding, we have demonstrated schedule-dependent modulation of 6-TG activity by MTX and have provided strong evidence that the mechanism of modulation is analogous to that more thoroughly worked out for the MTX-FUra interaction.

It is less clear what is the biochemical basis for 6-TG cytotoxicity. It has been reported (5) that the toxic effects ot 6-TG derive from its incorporation into cellular DNA. The results of the present study, while not conclusive, suggest that 6-TG incorporation into RNA, rather than DNA, is the most important event contributing to drug toxicity. Both the selective hydrolysis and the cesium sulfate gradient techniques gave the result that, under conditions where MTX potentiates the cytotoxic effects of 6-TG, there is a marked increase in the rate of incorporation of 6-TG into RNA and a dramatic suppression of 6-TG incorporation into DNA. It is possible that incorporation of abnormal bases into RNA could cause alterations in RNA function. This type of effect has been observed with other antimetabolites such as 5-azacytidine and FUra (10,11), though the contribution of 6-TG incorporation into RNA to cytotoxicity has not been previously established. While the increased 6-TG incorporation into RNA is understandable in terms of elevated levels of 6-TG nucleotides, the decreased incorporation of 6-TG into DNA is not as simple to explain. The suppression may, at least, be partly due to the inhibition of DNA synthesis caused by MTX; only direct measurements of the rate of DNA synthesis will reveal whether the differential rate of 6-TG incorporation into DNA is altered by MTX treatment. It is also interesting to note that 6-TGRP has been reported to inhibit several enzymes involved in de novo purine biosynthesis (2). This could provide an explanation not only for the decreased incorporation of 6-TG into DNA but also for a synergistic cytotoxicity with MTX

and 6-TG. The present experiments have not addressed this issue, and further work will be needed to clarify the relative contributions of the various effects of 6-TG to the ultimate lethality of the drug treatment.

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Table 1

Metabolism of 6-TG to 6-TG-Nucleotides

L1210 cells both with and without pre-exposure to 1 μM MTX were exposed to 0.5 μM (^{14}C)6-TG for 60 min, and the cellular levels of 6-TG and 6-TG-nucleotides determined as described under "Materials and Methods."[1]

Drug	pmol/10^6 cells				
	6-TG	TGMP	TGDP	TGTP	Total Nucleotide
6-TG	3.43	0.90	0.28	0.37	1.55
MTX→6-TG	3.36	1.60	0.32	0.48	2.40

[1] Results are representative of 4 separate experiments. The variation between experiments was less than ten percent.

Figure Legends

Fig. 1 The effect of methotrexate (MTX) on purine synthesis and 5-fluorouracil (5-FU) metabolism. Because of the depleted folates, purine synthesis ceases allowing the phosphoribosylpyrophosphate (PRPP), which would have been utilized for de novo purine biosynthesis, to be used for the conversion of 5-FU to 5-FUMP.

Fig. 2 Cytotoxicity of 6-TG in L1210 cells. L1210 leukemia cells were exposed for 3 hr to 6-TG either alone (●), simultaneously with 1 μM MTX (Δ), or following a 3 hr pre-epxosure to 1 μM MTX (o), and percent clone growth determined. Clonal growth of cells exposed to 1 μM MTX alone for 3 hr is illustrated by the dotted line. Results represent the mean of 4 separate experiments.

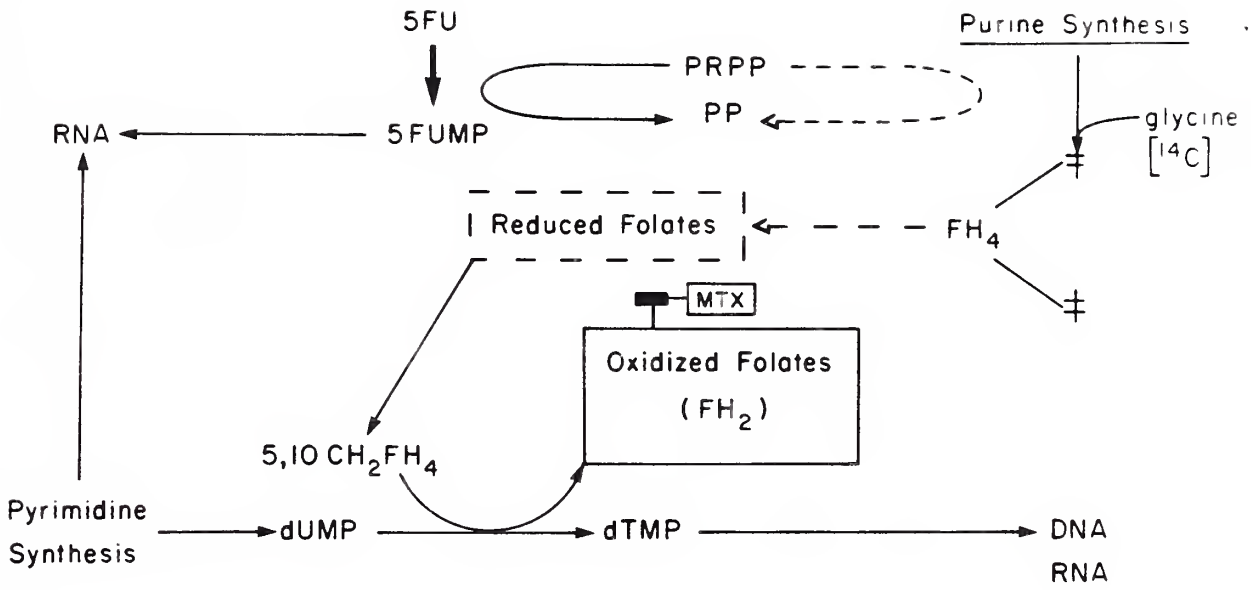
Fig. 3 Accumulation of 6-TG in intracellular acid-soluble pools. L1210 cells were exposed to 1 μM (^{14}C)6-TG for 1 to 180 minutes and the acid-soluble intracellular radioactivity measured. Cells were exposed either to 6-TG alone (●) or 6-TG following a 3 hr pretreatment with 1 μM MTX (o). Results are representative of 4 separate experiments.

Fig. 4 Incorporation of 6-TG into RNA: selective hydrolysis assay. L1210 cells were exposed to 1 μM (^{14}C)6-TG for 1 to 180 minutes and alkali-hydrolyzable, PCA-precipitable radioactivity determined (see Methods). Cells were exposed to 6-TG alone (●) or to 6-TG following a 3 hr pretreatment with 1 μM MTX (o). Results are representative of 4 separate experiments.

Fig. 5 Incorporation of 6-TG into DNA: selective hydrolysis technique. L1210 cells were exposed to 1 μM (^{14}C)6-TG for 1 to 180 minutes, and PCA-precipitable, hot acid-hydrolyzable radioactivity determined (see Methods). Cells were exposed to 6-TG alone (●) or to 6-TG following a 3 hr pretreatment with 1 μM MTX (o). Results are representative of 4 separate experiments.

Fig. 6 Incorporation of 6-TG into RNA: cesium sulfate gradient technique. L1210 cells were exposed to 1 μM (^{14}C)6-TG for 10 to 180 minutes, the nucleic acid isolated and separated into DNA and RNA fractions by cesium sulfate density gradient centrifugation, and the corresponding radioactivity determined (see Methods). Cells were exposed either to 6-TG alone (●) or to 6-TG following a 3 hr pretreatment with 1 μM MTX (o).

Fig. 7 Incorporation of 6-TG into DNA: cesium sulfate gradient technique. L1210 cells were exposed to 1 μM (^{14}C)6-TG for 10 to 180 minutes, the nucleic acid isolated and separated into DNA and RNA fractions by cesium sulfate density gradient centrifugation, and the corresponding radioactivity determined (see Methods). Cells were exposed to either 6-TG alone (●) or to 6-TG following a 3 hr pretreatment with 1 μM MTX (o).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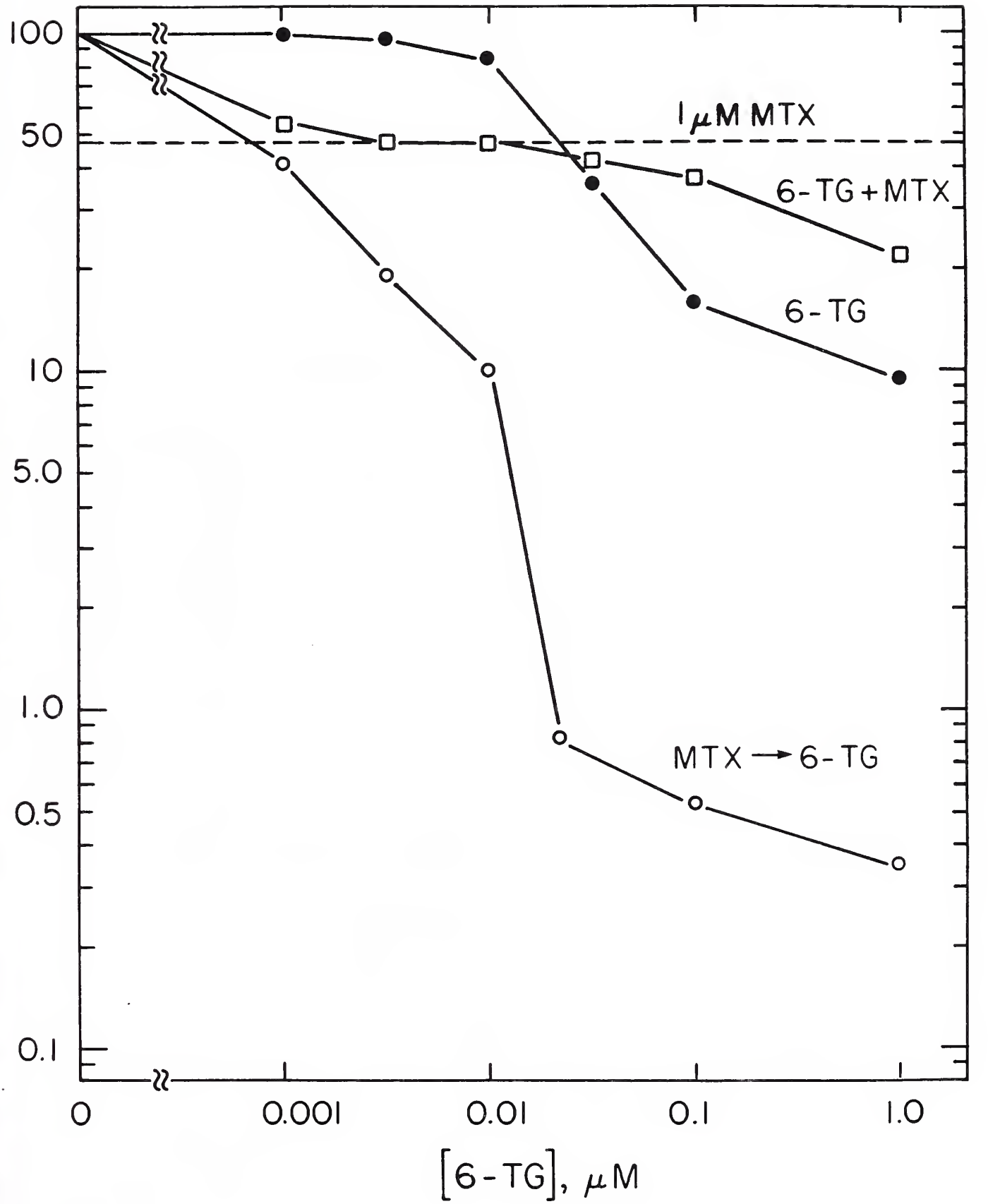
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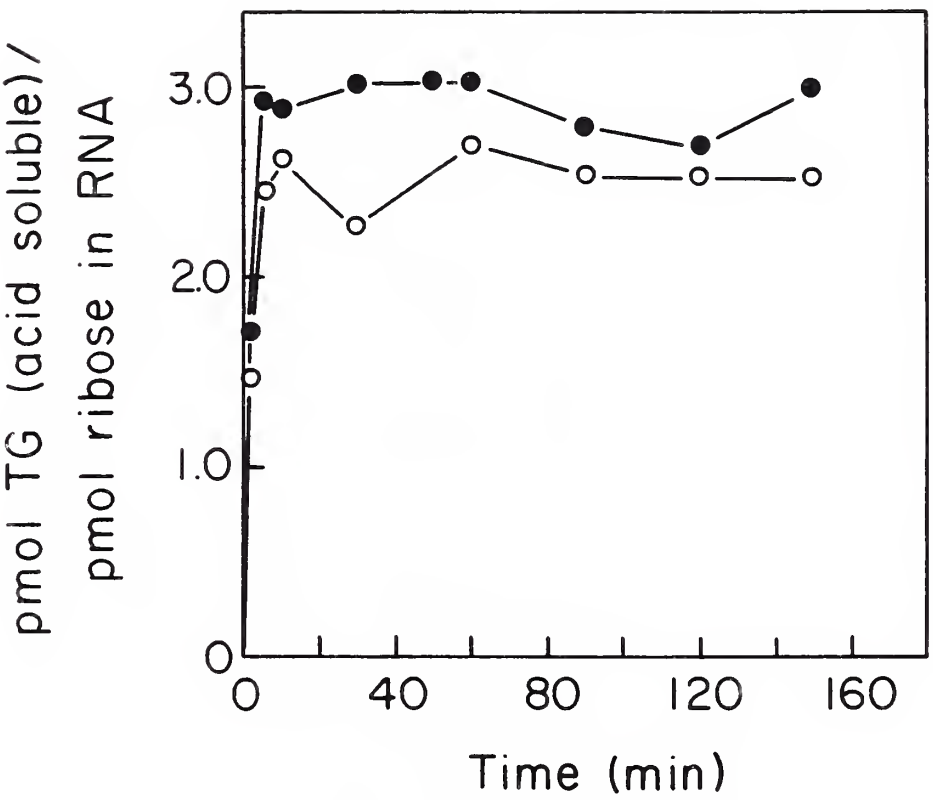
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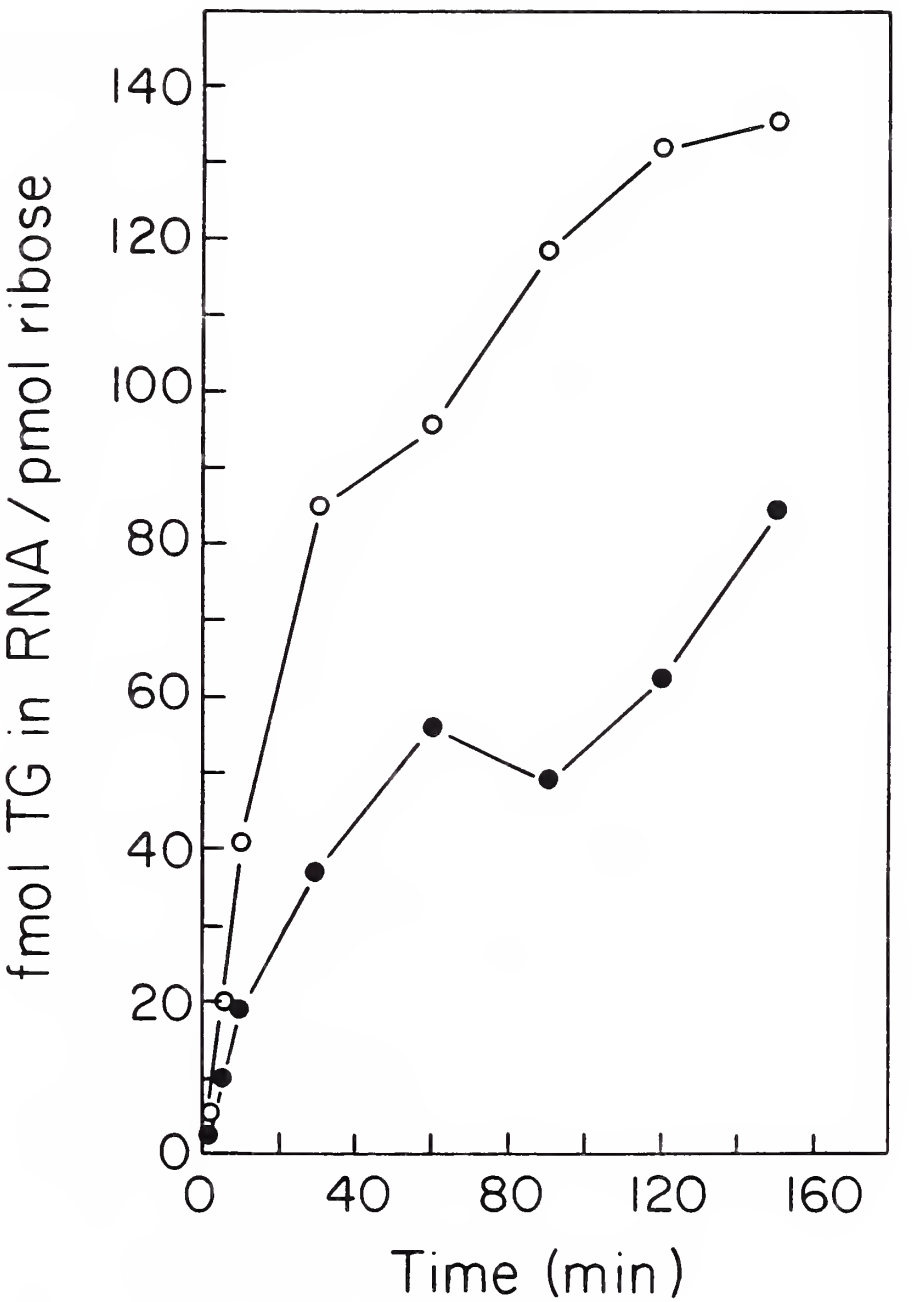
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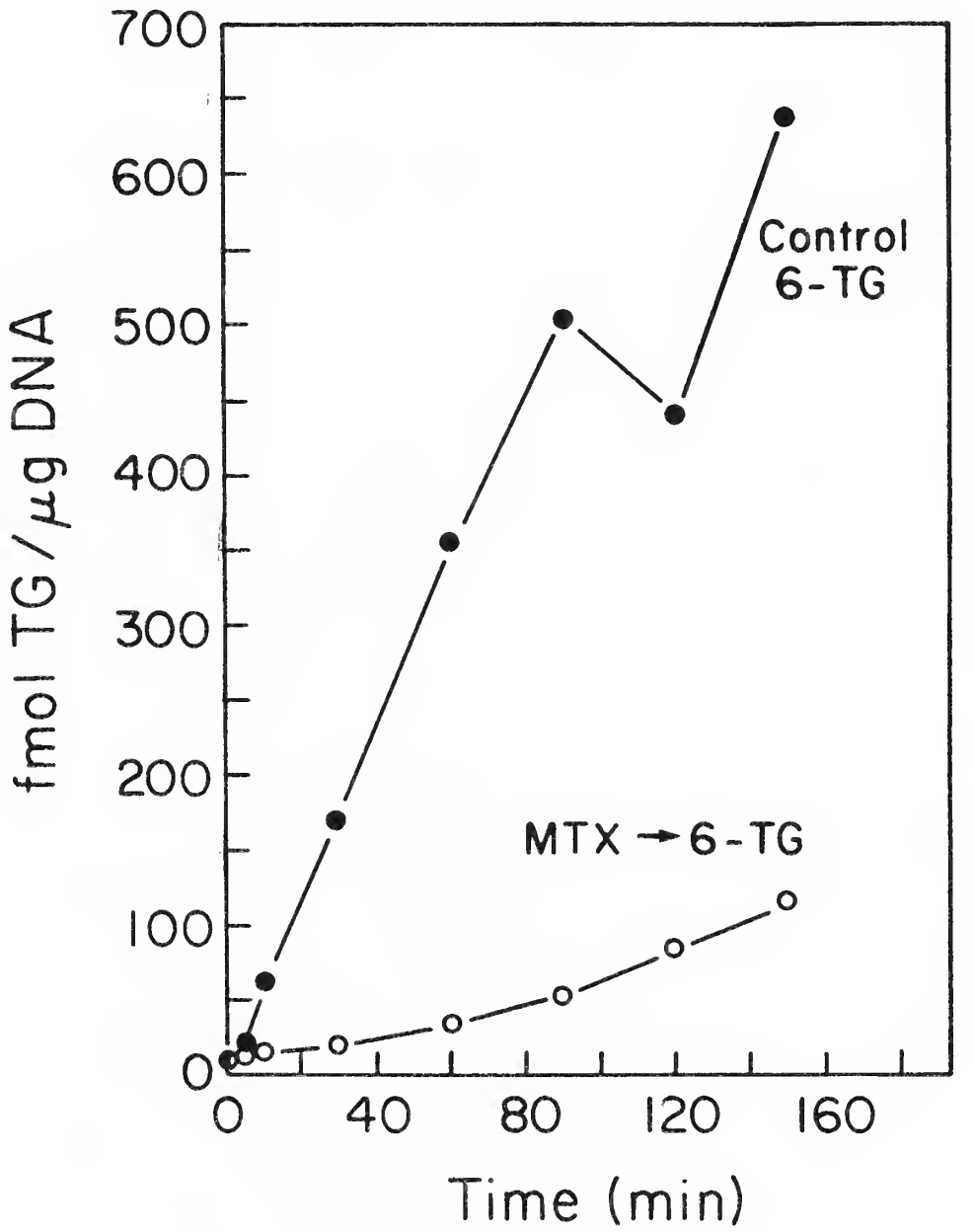
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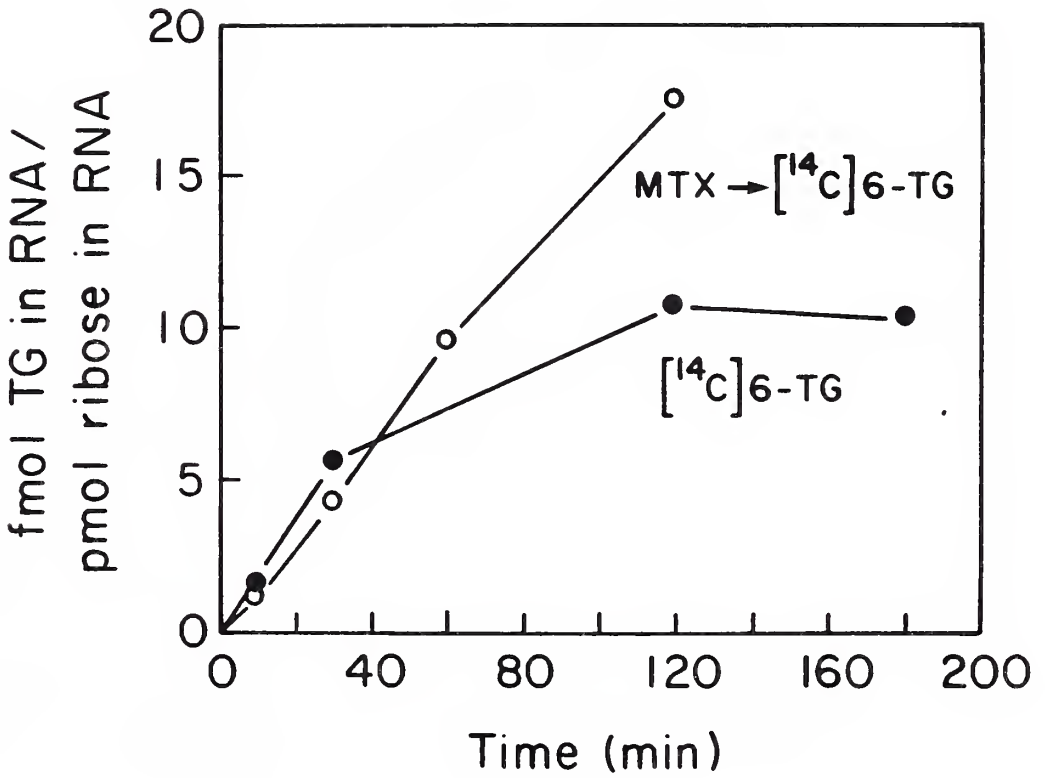
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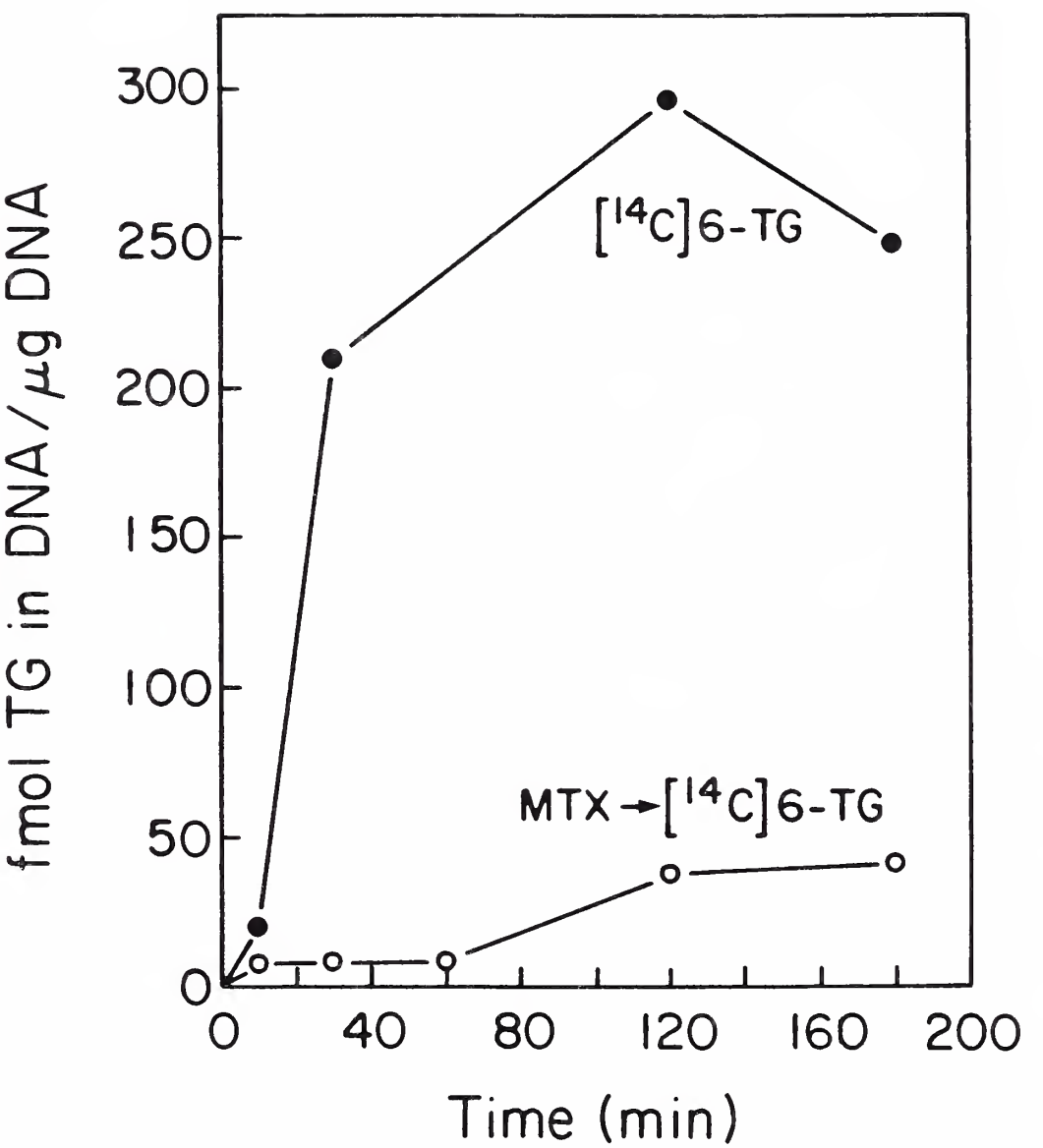
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